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P67091

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R S R LIMITED  
Avenue Park,  
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Patents ADP number (*if you know it*)

If the applicant is a corporate body, give the  
country/state of its incorporation

England and Wales

6021582001

4. Title of the invention

Assays for TSH Receptor Autoantibodies

5. Name of your agent (*if you have one*)

"Address for service" in the United Kingdom  
to which all correspondence should be sent  
(*including the postcode*)

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1600005

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Description

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Claim(s)

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*Hedley W. Austin* - Dykes

Date

5th June 1998

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01792 474327

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### Assays for TSH Receptor Autoantibodies

The present invention is concerned with assays (kits and analytical methods) for detecting or monitoring TSH Receptor Autoantibodies.

#### Background

The hyperthyroidism which is associated with Graves' disease is known to be due to autoantibodies to the thyroidal receptor for thyroid stimulating hormone (TSH). The autoantibodies bind to the receptor and mimic the actions of the natural ligand (TSH), thereby causing the gland to produce high levels of thyroid hormones (as described in Endocrine Reviews 1988, Vol 9, No. 1, pages 106 to 117).

The detection or monitoring of TSH receptor autoantibodies (TRAb) is important in the diagnosis and management of Graves' disease and currently two types of assay are used, namely:

- (a) competitive binding assays which measure the ability of TRAb to inhibit the binding of  $^{125}\text{I}$ -labelled TSH to preparations of TSH receptor; and
- (b) bioassays which measure the ability of TRAb to stimulate thyroid cells (or other cells transfected with the TSH receptor gene) in culture.

Currently, competitive binding assays (type (a) above) are more widely used, because bioassays of the type mentioned in (b) above are expensive, time-consuming, require highly skilled staff and are unsuitable for wide routine use. In current competitive binding assays, test serum samples ( $50\mu\text{l}$ ) are generally incubated with detergent solubilised porcine TSH receptor ( $50\mu\text{l}$ ). TRAb present in the test sera bind to the TSH receptor during this incubation.  $^{125}\text{I}$ -labelled TSH is then added and the incubation continued. During this second incubation, the labelled TSH binds to TSH receptors not already occupied by TRAb. Finally,  $^{125}\text{I}$ -labelled TSH bound to the receptor is separated from free labelled TSH by addition of polyethylene glycol (PEG), which precipitates the receptor bound TSH but not the free TSH. The radioactivity in the precipitates (separated by centrifugation) is then counted. In the assay, TRAb in test samples inhibits the binding of labelled TSH to the TSH receptor and this results in a lowering of the radioactivity in the precipitates. Assay results can be expressed as an index of inhibition of labelled TSH binding or by use of a set of assay calibrators.

The main limitations of this conventional assay are as follows:

- (a) The assay measures competition between labelled TSH and the TSH receptor and may not detect TRAb which bind well to the receptor but in such a way as not to inhibit TSH binding strongly.
- (b) The assay uses polyethylene glycol to separate receptor bound and free labelled TSH. This results in co-precipitation of all the serum immunoglobulins and the formation of a relatively large pellet. Although the pellet can be counted for radioactivity, it is not a suitable preparation to detect TSH (or other proteins or peptides) labelled with non-radioactive substances such as enzymes or chemiluminescent materials. This is because the serum components in the pellet interfere with such processes as light emission.

In addition, the use of PEG precipitation necessitates the use of centrifugation and this is a time-consuming and cumbersome procedure unsuitable for automation.

The present invention concerns procedures and kits for assays for TRAb of the competitive binding type mentioned above. The invention also concerns procedures and kits for TRAb assays of the direct binding type, in which a direct interaction between the receptor and TRAb is used.

The invention comprises the use of antibodies in order to label or immobilise a TSH receptor, the immobilised or labelled TSH receptor being such that it retains its ability to bind TSH and/or TRAb.

The present invention preferably concerns the use of a monoclonal (or polyclonal) antibody to the TSH receptor, which is bound strongly to the receptor at a site distinct from the part of the receptor which binds TSH and TRAb. The antibody can bind to the receptor strongly at the same time as TSH or TRAb and can be used to alleviate many of the limitations of the current TRAb assay method.

In a first embodiment of the invention, the antibody can be immobilised on a solid phase (such as a plastic tube or plastic plate, or magnetic or non-magnetic particles) using standard procedures. This solid phase can then be used instead of PEG to separate labelled TSH bound to the TSH receptor from free labelled TSH. The TSH (or similar ligand) can be labelled with isotopic or non-isotopic labels.

There are several applications of this first embodiment, of which the following are schematic examples:

(i) TSH receptor + labelled TSH  $\longrightarrow$  TSH receptor - labelled TSH

↑  
Binding inhibited  
by TRAb

↓  
separate from unbound labelled  
TSH by addition of immobilised  
receptor antibody (□-Ab)

↓  
□-Ab-TSH receptor-labelled TSH

(ii) □-Ab-TSH receptor + labelled TSH  $\longrightarrow$  □-Ab-TSH receptor-labelled TSH

↑  
Form this complex first      binding inhibited  
by TRAb

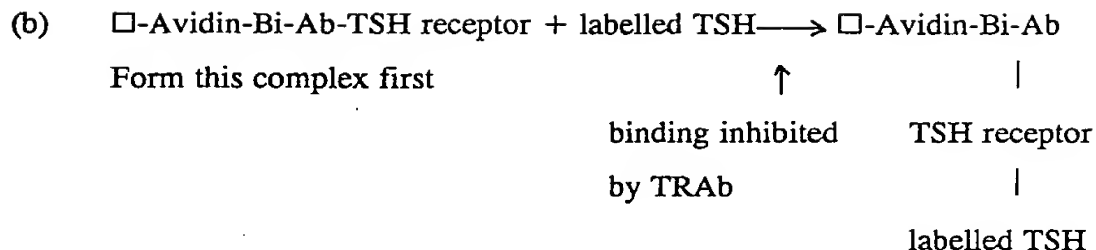
(iii) In (i) and (ii) above, the receptor Ab can be bound **indirectly** to the solid phase. For example, the Ab can be biotinylated and reacted with the solid phase which contains avidin or streptavidin:

(a) Bi-Ab-TSH receptor + labelled TSH  $\longrightarrow$  Bi-Ab-TSH receptor-labelled TSH complex

↑  
binding inhibited by TRAb

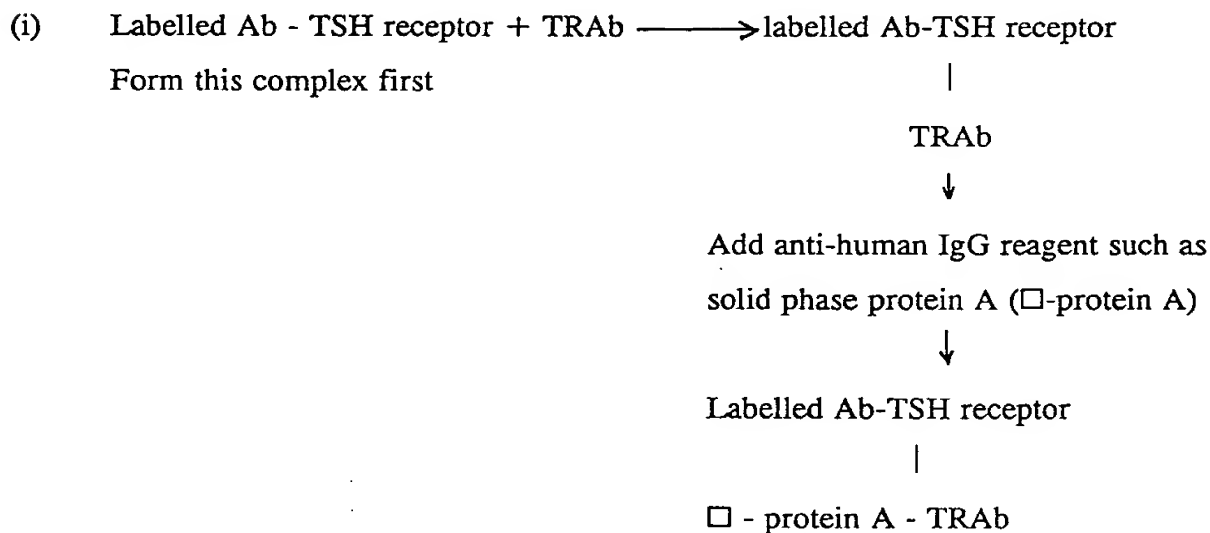
↓  
separate from unbound  
labelled TSH by addition  
of immobilised avidin or  
streptavidin (□-Avidin)

↓  
□-Avidin-Bi-Ab-TSH receptor-labelled TSH



In a second embodiment of the invention, the antibody can be labelled directly with an isotopic label such as  $^{125}\text{I}$ , or with a non-isotopic label such as an enzyme, dye, or chemiluminescent compound. Alternatively, the antibody may be labelled indirectly using, for example, the avidin-biotin system. The labelled antibody can then be used to label the TSH receptor itself and this complex of receptor and antibody can then be used to detect and/or monitor TRAb (either qualitatively or quantitatively).

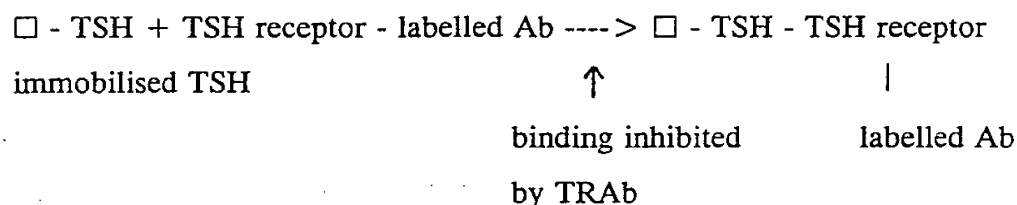
Examples of this approach are described schematically as follows:



In this system, increasing amounts of TRAb in a test sample will result in increasing amounts of labelled Ab in the final complex.



- (ii) This example depends on the coupling of TSH (or similar ligand) directly or indirectly to a solid phase ( $\square$ -TSH) receptor labelled with the antibody (which is itself labelled with an isotopic or non-isotopic label), which can bind to this immobilised TSH (or similar ligand). This binding will be inhibited by TRAb, as illustrated below:-



The schemes described above with reference to the first and second embodiments of the invention illustrate certain advantages of the present invention. The immobilised antibody may be used to label or immobilise the TSH receptor in such a way that the receptor retains its ability to bind TSH and/or TRAb. Alternatively, the immobilised antibody may be used for the monitoring of TRAb (or other ligands which interact with the TSH receptor) in patient sera, or for purification of the TSH receptor.

In particular, the ability to label the TSH receptor using the antibody permits monitoring of the direct interaction of TRAb with the TSH receptor as in the second embodiment of the invention described above. Furthermore, the present invention allows the immobilisation of the receptor before, during or after interaction with TSH and TRAb. This ability to immobilise the receptor can be used to create new TRAb assays which do not depend on PEG and/or isotopic labels. Such assays may be suitable for automation and immunochromatographic systems.

#### Detailed description of preferred features of the invention

The TSH receptor (TSHR) is present in very low numbers on the surface of thyrocytes (about  $10^3$  receptors per cell) which has made the receptor very difficult to purify from native sources (as described in "Baillière's Clinical Endocrinology and Metabolism", 1997, Vol II, pages 451 to 474 - Sanders).

In contrast, recombinant TSHR can be expressed in mammalian cells (for example, in Chinese hamster ovary (CHO) cells) at much higher levels of about  $10^5$ - $10^6$  receptors per cell (Sanders). In addition, recombinant TSHR preparations produced in non-thyroid cells are not contaminated with other thyroid autoantigens such as thyroglobulin or thyroid peroxidase (Springer Seminars in Immunopathology, 1993, pages 309-318 - Furmaniak).

Recombinant TSHR preparations produced in mammalian cells are the only ones which show TSH and TRAb binding characteristics similar to that of the native receptors. Such binding characteristics are not, for example, produced in yeast, insect cells or bacteria. This is because of a very complex relationship between the TSHR's structure and the TSH/TRAb binding sites. The TSHR's post-translational processing and folding of the "mature" protein is best achieved in the mammalian cell environment (see Sanders).

Purification of large amounts of recombinant TSHR from mammalian cells with its TSH and TRAb binding activities intact has not been reported. One of the major problems is loss of TSH/TRAb binding activity following binding with mouse monoclonal antibodies interacting with the extracellular part of the TSHR (Sanders). Also, to date, development of new and convenient strategies for routine measurement of TRAb has not been successful.

Preferred features of the present invention are illustrated by the following non-limiting detailed worked examples.

## EXAMPLES

### 1. Cloning of porcine TSHR cDNA

RNA was extracted from porcine thyroid tissue using the guanidinium thiocyanate-phenol chloroform method (Chomczynski and Sacchi, Anal. Biochem. Vol 162. 1987, pages 156 to 159). mRNA was purified from total RNA using a Dynal bead mRNA purification kit (Dynal, Wirral L62 SAZ UK). This mRNA was used to make a cDNA library using ZapExpress cDNA Gigapack Cloning Kit III (Stratagene Ltd., Cambridge CB4 4DF UK). Four degenerate oligonucleotides were made to the known TSHR sequences (mouse, rat, human, dog and bovine) and two fragments of porcine TSHR amplified using PCR. These were sequenced to verify their homology with TSHR cDNA and used to screen the cDNA library for full-length porcine TSHR clones. Three full-length clones were obtained and fully sequenced.

## 2. Expression of porcine recombinant TSHR protein in CHO cells

An ATG start codon in the 5' untranslated region (5'UTR) of the full-length pTSHR cDNA was removed by PCR and the cDNA cloned into pcDNA 3.1 (+) (Invitrogen BV, 9351 NV Leek, The Netherlands). DNA encoding the full-length TSHR was transfected into CHO cells (CHO-K1 from ECACC, Porton Down SP4 OJG UK) by electroporation. Clones expressing TSHR were detected using  $^{125}\text{I}$ -TSH binding directly to cells growing on 24-well plates. The clones showing highest TSH binding were expanded and recloned twice by limiting dilution. One stable cell line expressing about  $4 \times 10^5$  TSHR per cell was chosen for expansion and production of recombinant TSHR.

## 3. Preparation of detergent solubilised recombinant porcine TSHR

CHO cells expressing TSHR were grown to confluence, detached from roller bottles and the cell pellet washed with ice-cold 50 mM NaCl, 10 mM Tris-HCl, pH 7.5 containing 1 mM phenylmethylsulfonylfluoride (PMSF), then homogenised in the same buffer. The cell membranes after centrifugation at 12000g for 30 min at 4°C were solubilised in the same buffer (4 ml of buffer for approximately  $4 \times 10^8$  cells) as used for homogenisation except for addition of 1% Triton X-100. The solubilised receptor preparations were centrifuged at 90,000g for two hours at 4°C and the supernatant stored at -70°C.

## 4. Expression of the C-terminal end of the porcine TSHR protein

The expression in *Escherichia coli* as a fusion protein with glutathione S-transferase (GST) was carried out using standard protocols (as described in Journal of Molecular Endocrinology (1998) Vol 20, pages 233-244 - Oda). The 3' end of cDNA (1809 to 2295 bp) coding for the last 160 amino acids was cloned in frame with the GST fusion protein in pGEX2T vector (Pharmacia Biotech, St. Albans AL1 3AW UK). An overnight culture of *E. coli* (strain UT580) transformed with pGEX-2T/TSHR plasmids was diluted 1/5 into 2 x YTG medium (16 g Tryptone, 10g yeast extract, 5 g NaCl, 20 g glucose per litre, pH 7.0), incubated for 3 hours at 30°C. Thereafter, isopropyl-3-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM in order to induce protein expression, followed by incubation for a further three hours. The bacterial pellets were resuspended in PBS (8 g NaCl, 0.2 g KCl, 1.44 g  $\text{Na}_2\text{HPO}_4$  and 0.24 g  $\text{KH}_2\text{PO}_4$  per litre, pH 7.4) containing 1% Triton X-100 and sonicated

three times for one minute on ice. The inclusion bodies were pelleted, washed in 4M urea, solubilised in 8M urea and separated on 9% polyacrylamide gels (SDS-polyacrylamide electrophoresis, SDS-PAGE) under reducing conditions. The TSHR/GST fusion proteins (mol. wt. 44 kDa) were electroeluted from polyacrylamide gel slices in 0.1M NaHCO<sub>3</sub> and 0.1% SDS pH 7.8, dialysed against 50 mM Tris-HCl pH 8.0 and stored in aliquots at -70°C.

#### 5. Preparation and purification of monoclonal antibodies to the porcine TSHR

Electroeluted TSHR/GST protein was used to immunise BALB C mice (50 µg per mouse per injection) until the titre of antibody to the TSHR was high. The TSHR antibody level in mouse sera was tested using immunoprecipitation assay based on <sup>35</sup>S-labelled TSHR produced in an in vitro transcription/translation system (see the method described in Journal of Clinical Endocrinology and Metabolism, Vol 82 (1997) No. 4, pages 1288-1292 - Prentice).

Mouse spleen cells were fused with mouse myeloma cell line (X63-Ag8.653 from ECACC) and cloned to produce stable hybridomas secreting the TSHR antibody using standard techniques (Oda). The antibody 4E31 was found to precipitate <sup>35</sup>S-TSHR in the immunoprecipitation assay and to react well with the TSHR in western blotting analysis.

4E31 was purified from hybridoma culture supernatants by chromatography on a Prosep-A (Bioprocessing, Consett DH8 6TJ UK) column. Fab<sub>2</sub> fragments were obtained following digestion with pepsin (pepsin concentration of 1 mg/ml in 70 mM sodium acetate, 50 mM NaCl pH 4.0, IgG to enzyme ratio of 5:1) and chromatography on Prosep A to adsorb intact IgG. 4E31 Fab<sub>2</sub> preparations of about 1 mg/ml were stored at -70°C in aliquots.

#### 6. TSH receptor binding characteristics of the 4E31 antibody

Solubilised recombinant porcine TSH receptor was incubated with <sup>125</sup>I-labelled TSH to form a <sup>125</sup>I-labelled TSH-TSH receptor complex. The 4E31 IgG (or control IgG) was immobilised by linking it to magnetic latex beads and these beads (100 µl; 1 µg of 4E31) were incubated with the preformed <sup>125</sup>I-labelled TSH-TSH receptor complex (100 µl). After 1 hr at 37°C, the beads were separated on a magnet, washed and counted for <sup>125</sup>I. A sample of the <sup>125</sup>I-labelled TSH-TSH receptor complex was precipitated with PEG to determine the amount of free labelled TSH present. Table 1 shows the results obtained:

Table 1

Sample	cpm Bound to Beads
4E31 IgG beads plus:-	
(a) labelled TSH-TSH receptor complex	16,374
(b) labelled TSH only	2,118
Control IgG beads plus labelled TSH-TSH receptor complex	2,433
Labelled TSH-TSH receptor complex precipitated by PEG	16,499
Total cpm in 100 $\mu$ l of labelled complex	37,073

(Control IgG used was a mouse monoclonal antibody to glutamic acid decarboxylase)

These studies indicated clearly that 4E31 could bind to the TSH receptor at the same time as TSH. Further, 4E31 coupled to a solid support could be used to separate TSH bound to the TSH receptor from free TSH with results similar to those obtained with PEG.

Table 2 shows that in the presence of individual healthy human donor sera, about 55 % of the labelled TSH added bound to tubes coated with 4E31 and recombinant porcine TSH receptor (according to the first embodiment of the invention, scheme ii above). Similar binding was observed in the case of sera from patients with Hashimoto's thyroiditis and patients with systemic lupus erythematosus (SLE). However with sera from the 5 patients with Graves' disease, there was markedly less labelled TSH binding (about 35 %). All 5 sera contained readily detectable amounts of TSH receptor autoantibody as judged by inhibition of TSH binding to native porcine TSH receptor and separation of receptor bound and free TSH with PEG.

Table 2 Binding of labelled TSH to plastic tubes coated with 4E31 (Fab)<sub>2</sub> followed by recombinant porcine TSH receptor - effect of different sera.

Sample	cpm bound	inhibition of TSH binding (%)	
		coated tube method (recombinant receptor)	PEG method (native receptor)
Individual normal sera:-			
1	15,171		
2	15,209		
3	15,480		
4	14,768		
5	15,496		
Individual Graves' sera:-			
1	10,132	34	27
2	9,575	37	31
3	12,495	18	21
4	11,478	25	11
5	9,163	40	48
Individual Hashimoto sera:-			
1	14,659		
2	15,215		
3	14,603		
4	15,026		
5	15,370		
Individual SLE sera:-			
1	15,711		
2	15,261		
3	15,091		
4	15,386		
5	15,403		
Total cpm	27,420		

Table 3 shows similar results with tubes coated with native (i.e. non-recombinant) porcine TSH receptor via 4E31. Again, the results obtained with the coated tube assay are similar to those obtained with the PEG separation method.

Table 3 Binding of labelled TSH to plastic tubes coated with 4E31 (Fab)<sub>2</sub> followed by native detergent solubilised porcine TSH receptor.

Sample	inhibition of TSH binding (%) with:-	
	native porcine TSH receptor bound to plastic tubes (via 4E31 (Fab) <sub>2</sub> )	native porcine TSH receptor PEG precipitation assay
Individual normal sera:-		
a	1.5	-0.62
b	2.2	3.2
c	-5.0	3.9
d	1.8	3.2
Graves sera:-		
6	74	71
7	75	72
8	81	76
9	70	73
10	77	88
11	66	83
12	88	72
13	80	76
14	57	70
15	90	86
16	55	72
17	81	68

The inhibiting effect on  $^{125}\text{I}$ -TSH binding shown in tables 2 and 3 was dependent on the concentration of TRAb in the serum. As shown in Table 4, increasing amounts of TRAb standard preparation (thyroid stimulating antibody 1st International Standard 90/672) had a dose dependent effect on TSH binding inhibition.

Table 4

TRAb standard dilution (mU/ml)	native porcine TSH receptor (PEG separation method) - inhibition of TSH binding (%)	4E31/recombinant porcine TSH receptor coated tubes - inhibition of TSH binding (%)
0	0	0
1.25	5.1	5.1
2.5	9.9	12.0
5	16.2	17.0
10	28.4	31.7
20	44.5	51.6
40	69.1	74.8



### Direct precipitation of $^{125}\text{I}$ -4E31 Fab<sub>2</sub>-TSH complexes

Another example of the invention (according to the second embodiment of the invention, scheme (i) above) is an assay in which 4E31 (Fab)<sub>2</sub> is labelled with  $^{125}\text{I}$  and reacted with the TSH receptor. This complex is then incubated with TSH receptor autoantibodies in patients' sera and the resulting termolecular complex precipitated by addition of solid phase protein A. An example is shown in Table 5.

**Table 5** Reaction between recombinant porcine TSH receptor labelled with  $^{125}\text{I}$ -4E31 (Fab)<sub>2</sub> and TSH receptor autoantibodies.

Sample	cpm bound
<b>Labelled receptor plus:-</b>	
Individual normal serum:-	
e	1,994
f	2,041
90/672 reference material (mU/ml):	
1.25	2,715
2.5	2,958
5	3,358
10	4,202
20	5,868
40	8,949
Graves' serum:-	
18	12,326
19	13,225
<b>Total cpm</b>	<b>48,428</b>

Graves' sera 18 and 19 gave TSH binding inhibition values of 31% and 73% respectively in the PEG precipitation method using native porcine TSH receptor.

4E31 could be labelled with biotin, bound to streptavidin coated tubes and then reacted with porcine TSH receptor - see the first embodiment of the invention, Scheme (iii) b. Receptor immobilised in this way readily bound  $^{125}\text{I}$ -labelled TSH and this binding was inhibited by TSH receptor autoantibodies in patient sera. An example is shown in Table 6.

**Table 6**      Labelled TSH binding to TSH receptor - bound to streptavidin coated tubes via biotinylated 4E31 (Fab)<sub>2</sub> and effect of TRAb.

Serum Sample	$^{125}\text{I}$ -labelled TSH bound (cpm)
Individual healthy normal sera:-	
g	12,225
h	12,164
TRAb positive Graves' sera:-	
20 :	2,971
21	7,400
Total cpm	27,339